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Obstruction of polyubiquitination affects PTS1 peroxisomal matrix protein import

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Abstract

Pex4p is an ubiquitin-conjugating enzyme that functions at a late stage of peroxisomal matrix protein import. Here we show that in the methylotrophic yeast *Hansenula polymorpha* production of a mutant form of ubiquitin (Ub^{K48R}) has a dramatic effect on PTS1 matrix protein import. This effect was not observed in cells lacking Pex4p, in which the peroxisome biogenesis defect was largely suppressed. These findings provide the first indication that the function of Pex4p in matrix protein import involves polyubiquitination. We also demonstrate that the production of Ub^{K48R} in *H. polymorpha* results in enhanced Pex5p degradation. A similar observation was made in cells in which the *PEX4* gene was deleted. We demonstrate that in both strains Pex5p degradation was due to ubiquitination and subsequent degradation by the proteasome. This process appeared to be dependent on a conserved lysine residue in the N-terminus of Pex5p (Lys21) and was prevented in a $Pex5p^{K21R}$ mutant. We speculate that the degradation of Pex5p by the proteasome is important to remove receptor molecules that are stuck at a late stage of the Pex5p-mediated protein import pathway.

Keywords: Peroxisome; Protein translocation; PTS1 protein import; Ubiquitin; Yeast; *Hansenula polymorpha*

1. Introduction

Peroxisomal matrix proteins are synthesized in the cytosol and post-translationally translocated across the peroxisomal membrane. Most matrix proteins are sorted via a peroxisomal targeting signal type 1 (PTS1), which is present at the extreme C-terminus of the protein and consists of the tripeptide–Ser–Lys–Leu–COOH or a conserved variant thereof (reviewed by [1]). The PTS1 protein sorting process starts with recognition of newly synthesized PTS1 proteins by the PTS1 receptor Pex5p in the cytosol. This receptor–cargo complex is thought to associate with the peroxisomal membrane by binding to a proteinaceous docking machinery that consists of three interacting proteins, the peroxins Pex13p, Pex14p and Pex17p [1]. A putative translocation complex, comprising three RING-finger-containing peroxisomal membrane proteins (Pex2p, Pex10p and Pex12p), appears to be closely connected to the docking complex and is supposed to play a role in the translocation process. Both complexes are held tightly together via protein–protein interactions presumably via the peroxins Pex3p or Pex8p [2,3]. Recent evidence suggest that the entire receptor–cargo complex may actually be translocated across the peroxisomal membrane ("the extended shuttle model" of import [4]). It is believed that inside the organellar matrix Pex5p interacts with Pex8p, resulting in the dissociation of the cargo from the receptor [5,6]. The final step of the PTS1 protein import pathway is the recycling of Pex5p to the cytosol to enable a new import cycle. Peroxins thought to be involved in this stage of the process [7] are two AAA-type ATPases, Pex1p and Pex6p, and Pex4p, an ubiquitin-conjugating enzyme [8–10] that is anchored to the peroxisomal membrane via the peroxisomal membrane protein Pex22p [11].

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Ubiquitin-conjugating enzymes are involved in the modification of protein substrates by the covalent binding of ubiquitin (Ub), a peptide of 76 amino acids. Ub-conjugation involves three consecutive steps. In the first step Ub is activated in an ATP-dependent way by an Ub-activating enzyme, also designated E1. This enzyme transfers the activated Ub to an Ub-conjugating enzyme (E2), which finally acts in conjunction with an accessory Ub ligase (E3) that is involved in the recognition of the target protein (reviewed in [12,13]). This results in the formation of an isopeptide bond between the C-terminal glycine residue of Ub and the ε-amine group of a lysine residue in the substrate molecule. Often poly-Ub chains become conjugated to a substrate protein. Such chains are elongated by subsequent conjugation of additional Ub molecules to an internal lysine residue (mostly Lys48, occasionally another lysine residue of Ub [13]) in the previous Ub moiety that was already attached to the substrate protein.

Ubiquitination plays a role in several biological processes (e.g. the cell cycle, endocytosis, transcription, DNA repair, proteasomal degradation; see [13] and references therein). In some of these processes only a single Ub molecule is conjugated to a substrate molecule, whereas other functions involve polyubiquitination.

As yet it is still an enigma whether the sole E2 enzyme involved in peroxisome biogenesis, Pex4p, mono- or polyubiquitinates its substrate. The data presented in this paper provide the first indication that in the methylotrophic yeast *Hansenula polymorpha* the function of Pex4p in PTS1 protein import involves polyubiquitination. Additionally, also evidence is provided that defects in Pex4p functioning result in Pex5p degradation, a process that appears to involve a Pex4p-independent ubiquitination of Pex5p. We propose that the physiological function of this process is the removal of non-functional Pex5p molecules from the peroxisomal translocation machinery.

2. Materials and methods

2.1. Organisms and growth conditions

The *H. polymorpha* strains used in this study are all derivatives of NCYC495 and are listed in Table 1. The *pex4 pex5* double mutant was obtained by crossing the *pex4* (*leu1.1*) and *pex5* (*ura3*) mutants according to Gleeson and Sudbery [14]. Diploids were subjected to random spore analysis, and prototrophic segregants were subjected to complementation analysis to determine their genotypes.

Yeast cells were cultivated in batch cultures at 37 °C on (i) rich media, containing 1% yeast extract, 1% peptone and 1% glucose (YPD), (ii) selective minimal media, containing 0.67% Yeast Nitrogen Base without amino acids (DIFCO) supplemented with 1% glucose, or (iii) minimal medium [15], using glucose (0.5%), glycerol (0.5%), glycerol/methanol (0.1%+0.5%, respectively) or methanol (0.5%) as carbon sources, and methylamine (0.25%) or ammonium sulfate (0.25%) as nitrogen sources. When required, amino acids or uracil was added to a final concentration of 30 μg/ml. For growth on agar plates the media were supplemented with 1.5% agar. For the selection of zeocin-resistant transformants YPD plates containing 100 μg/ml zeocin (Invitrogen) were used.

Table 1
H. polymorpha strains used in this study

Strains	Characteristics	Reference
NCYC495 (*leu1.1*)	Wild type	[14]
NCYC495	Wild type, prototrophic	[14]
pex3 (*leu1.1*)	NCYC495 with deletion in *PEX3*	[34]
pex4 (*leu1.1*)	NCYC495 with deletion in *PEX4*	[10]
pex5 (*leu1.1*)	NCYC495 with deletion in *PEX5*	[33]
pex5 (*ura3*)	NCYC495 with deletion in *PEX5*	[35]
pex7 (*leu1.1*)	NCYC495 with deletion in *PEX7*	A. Koek et al., unpublished
pex8 (*leu1.1*)	NCYC495 with deletion in *PEX8*	R.J.S. Baerends et al., unpublished
pex4 pex5	Segregant of cross between *pex5* (*ura3*) and *pex4* (*leu1.1*)	This study
Ub.K48R	NCYC495 (*leu1.1*) with 2 copies of plasmid pX4-Ub.K48R integrated	This study
Myc-Ub.K48R	NCYC495 with 2 copies of plasmid pZ15-MycUb.K48R integrated	This study
pex3-Ub.K48R	*pex3* (*leu1.1*) with 2 copies of plasmid pX4-Ub.K48R integrated	This study
pex7-Ub.K48R	*pex7* (*leu1.1*) with 2 copies of plasmid pX4-Ub.K48R integrated	This study
pex8-Ub.K48R	*pex8* (*leu1.1*) with 2 copies of plasmid pX4-Ub.K48R integrated	This study
pex4-MycUb	*pex4* (*leu1.1*) with multiple copies of plasmid pX4-MycUb integrated	This study
PEX5.K21R	*pex5* (*leu1.1*) with 1 copy of plasmid pX12-PEX5.K21R integrated	This study
pex4 PEX5.K21R	*pex4 pex5* with 1 copy of plasmid pZ12-PEX5.K21R integrated	This study
PEX5.K21R MycUb.K48R	PEX5.K21R with 2 copies of plasmid pZ15-MycUb.K48R integrated	This study
pex4::P_{AOX}*PEX5*^{4c}	*pex4* overexpressing the *PEX5* gene under control of the *AOX* promoter	[10]
pex4::P_{AOX}*PEX5* ::MycUb	*pex4*::P_{AOX}*PEX5*^{4c} with 2 copies of plasmid pZ15-MycUb integrated	This study
pex4::P_{AOX}*PEX5* ::MycUb.K48R	*pex4*::P_{AOX}*PEX5*^{4c} with 2 copies of plasmid pZ15-MycUb.K48R integrated	This study
Ub-Leu-β-gal	NCYC495 with one copy of plasmid pZ5-Ub-Leu-β-gal integrated	This study
Ub.K48R Ub-Leu-β-gal	Ub.K48R with one copy of plasmid pZ5-Ub-Leu-β-gal integrated	This study

Table 2
Plasmids used in this study

Plasmid	Relevant properties	Reference
pHIPX4	Plasmid with *H. polymorpha AOX* promoter and *AMO* terminator; kan^R, *ScLEU2*	[36]
pHIPX4-HNBESX	Derivative of pHIPX4 with different polylinker	Lab collection
pHIPZ4	Plasmid containing *H. polymorpha AOX* promoter and *AMO* terminator regions; zeo^r ; amp^R	[37]
pHIPZ5	Plasmid containing *H. polymorpha AMO* promoter and *AMO* terminator regions; zeo^r ; amp^R	[38]
pHIPZ15	Plasmid containing *H. polymorpha DAS* promoter and *AMO* terminator regions; zeo^r ; amp^R	This study
YEp105	*E. coli*/*S. cerevisiae* plasmid with P_{CUP}-*MycUb*-T_{CYC} cassette; *ScTRP1*; amp^R	T. Sommer, MDC, Berlin, Germany
pUB203	*E. coli*/*S. cerevisiae* plasmid with P_{CUP}-*Ub.K48R*-T_{CYC} cassette; *ScTRP1*; amp^R	T. Sommer, MDC, Berlin, Germany
pUB-Leu-β-gal	Plasmid containing *Ub-Leu-β-gal* fusion gene ; amp^R	[19]
pRBG56-2	pBSK+ carrying a 2.5 kb *Sac*I fragment with *H. polymorpha PEX5* gene (Genbank U26678)	Lab collection
pX4-Ub.K48R	pHIPX4-HNBESX with *Ub.K48R* gene	This study
pX4-MycUb	pHIPX4-HNBESX with *MycUb* gene	This study
pHIPX4-PEX5	pHIPX4 with *H. polymorpha PEX5* gene	[35]
pZ15-MycUb	pHIPZ15 with *MycUb* gene	This study
pZ15-Ub.K48R	pHIPZ15 with *Ub.K48R* gene	
pZ15-MycUb.K48R	pHIPZ15 with *MycUb.K48R* gene	This study
pZ5-Ub-Leu-β-gal	pHIPZ5 with *Ub-Leu-β-gal* gene	This study
pX12-PEX5.K21R	Plasmid with P_{PEX5} -*PEX5.K21R*-T_{AMO} cassette; kan^R, *ScLEU2*	This study
pZ12-PEX5.K21R	Plasmid with P_{PEX5} -*PEX5.K21R*-T_{AMO} cassette; amp^R, zeo^R	This study

Escherichia coli DH5α was grown on LB medium [16]. When required, media were supplemented with ampicillin (100 μg/ml) or kanamycin (50 μg/ml).

2.2. Molecular techniques

Standard recombinant DNA techniques were carried out essentially according to Sambrook et al. [16]. The transformation of *H. polymorpha* cells [17] and site specific integration of single or multiple copies of plasmid DNA in the *H. polymorpha* genome [18] were performed as described. Correct integration in the *H. polymorpha* P_{AOX}, P_{DAS}, P_{AMO} and P_{PEX5} genomic regions was checked by Southern blot analysis using the ECL direct nucleic acid labeling and detection system according to the instructions of the supplier (Amersham Corp., Arlington Heights, IL). Preparative PCR was performed using *Pwo* polymerase according to the instructions of the supplier (Roche, Diagnostics Gmbh, Almere, The Netherlands).

2.3. Plasmid constructions

The plasmids and primers used in this study are listed in Tables 2 and 3, respectively. For the construction of plasmids pX4-MycUb and pX4-Ub.K48R, the 280 bp and 240 bp *Eco*RI–*Asp*718I (blunted) fragments from YEp105 and pUB203, respectively, were inserted between the *Eco*RI and *Sma*I sites of pHIPX4-HNBESX. For stable integration of the expression cassettes into the *H. polymorpha* genome, the plasmids were linearised with *Stu*I in the P_{AOX} region and transformed into various *H. polymorpha* strains (see Table 1).

To allow the expression of genes under control of an alternative, strong, methanol-inducible promoter, the vector pHIPZ15 was constructed containing the promoter region of the *DAS* gene encoding dihydroxyacetone synthase (Genbank accession number E00790). For this purpose the *DAS* promoter region was amplified with primers DHAS1 and DHAS2 using *H. polymorpha* NCYC495 genomic DNA as template. The resulting 1.5 kb PCR product was digested with *Bam*HI and cloned in vector pHIPZ4, digested with *Not*I (blunted) and *Bam*HI, thereby replacing the P_{AOX} in this plasmid.

For the construction of pZ15-MycUb and pZ15-Ub.K48R, the 1.5 kb *Nhe*I (blunted)–*Bam*HI fragments from

Table 3
Primers used in this study

Primers	Sequence
DHAS1	5′ GCT CGA GAG CCG TGG AAC AG 3′
DHAS2	5′ AGA GGA TCC GGG AAG AAA AGA CAG AGA TG 3′
PEX5-K21R	5′ ATG CTG CGA CTG TCT GAA AAA CTG CGC 3′
PEX5-return	5′ CTT TCG GCC TCG TTC ATA GC 3′
UbGal-start	5′ CGA GAT CTA TGC AGA TTT TCG TCA AGA CTT TG 3′
UbGal-stop	5′ GCG CGC ATG CTT ATT TTT GAC ACC AGA CCA ACT GG 3′
Universal M13/pUC	5′ GTA AAA CGA CGG CCA GT 3′

pX4-MycUb and pX4-Ub.K48R, respectively, were ligated into *Sal*I (blunted)–*Bam*HI digested pHIPZ15. For the construction of pZ15-MycUb.K48R, the 2.4 kb *Hpa*I–*Asp*718I fragment of pZ15-Ub.K48R was inserted into *Hpa*I+*Asp*718I-digested pZ15-MycUb. Plasmids pZ15-MycUb and pZ15-MycUb.K48R were linearised with *Bst*EII in the P_{DAS} region to enable integration in the *H. polymorpha* genome (Table 1).

Plasmid pZ5-Ub-Leu-β-gal was constructed by amplification of the *Ub-Leu-LacZ* fusion gene using primers UbGal-start and UbGal-stop and the plasmid pUB-Leu-β-gal as template [19]. The resulting 3.4 kb PCR product was digested with *Bgl*II and *Sph*I, and subsequently ligated in pHIPZ5 digested with *Bam*HI and *Sph*I. The resulting plasmid pZ5-Ub-Leu-β-gal was linearised with *Bsi*WI in the P_{AMO} region to enable the integration in the *H. polymorpha* genome (Table 1).

Plasmid pX12-PEX5.K21R was constructed by a two-step PCR approach: First, a 659 bp PCR fragment containing the promoter and part of the *PEX5* coding region including the Lys21Arg mutation was obtained with primer PEX5-K21R and the universal M13/pUC sequencing primer using plasmid pRBG56-2 as template. After purification, the PCR fragment was used as primer in a second PCR with primer PEX5-return using pRBG56-2 as template. The resulting 754 bp fragment was digested with *Not*I and *Xho*I, and was inserted into plasmid pHIPX4-PEX5, thereby replacing the *AOX* promoter and the 5′ end of the coding region of WT *PEX5*. Subsequently, the resulting plasmid, pX12-PEX5.K21R, was digested with *Apa*I in the P_{PEX5} region and inserted in the genome of *H. polymorpha*. For the construction of plasmid pZ12-PEX5.K21R, a 3156 bp *Not*I–*Nhe*I fragment from pX12-PEX5.K21R was inserted between the *Not*I and *Xba*I sites of plasmid pHIPZ4. Subsequently, the resulting plasmid pZ12-PEX5.K21R was linearised with *Bgl*II to enable integration in the *H. polymorpha* genome.

2.4. Biochemical methods

Crude extracts of *H. polymorpha* cells were prepared by the TCA method as described previously [20]. SDS-PAGE and Western blotting were performed by established methods. In all experiments equal amounts of protein were loaded per lane, which was controlled by Ponceau S staining of blots prior to detection. Blots were decorated using specific antibodies against various *H. polymorpha* proteins using either the Protoblot immunoblotting system (Promega Biotec) or the BM Chemiluminiscence Western Blotting kit (Roche, Diagnostics Gmbh, Almere, The Netherlands).

To block the function of the proteasome, the inhibitor MG132 was used [20]. *H. polymorpha* cells were grown in glycerol-containing medium to an OD_{600} of approximately 1. Subsequently, to one half of the culture, the proteasome inhibitor MG132 (Calbiochem, Omnilabo International b.v., The Netherlands) in DMSO was added to a final concentration of 50 μM. To the other half of the culture, DMSO was added as a control. Cells were allowed to resume growth at 37 °C for 1 h. Samples corresponding to 3 OD_{600} units of cells were taken and analyzed by Western blotting.

For β-galactosidase activity measurements, *H. polymorpha* cells were grown on glycerol/methanol containing media to the end of the logarithmic growth phase. After harvesting the cells, crude extracts were prepared using glass beads and β-galactosidase activities were determined according to Miller [21]. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad GmbH, Munich, Germany).

2.5. Electron microscopy

Whole cells were fixed and prepared for electron microscopy and immunocytochemistry as described previously [22]. Immunolabeling was performed on ultrathin sections of unicryl-embedded cells, using specific antibodies against alcohol oxidase (AO) and gold conjugated goat-anti-rabbit antibodies [22].

3. Results

3.1. Ub^{K48R} production affects PTS1 matrix protein import in H. polymorpha

Polyubiquitination via lysine 48 (Lys48) in Ub is thought to be the major type of poly-Ub conjugation to target proteins [13]. To analyze whether the function of Pex4p in PTS1 protein import involves poly-Ub chains, linked via Lys48 in Ub, we aimed to obstruct the formation of such poly-Ub chains in *H. polymorpha* by the production of a mutant form of Ub, Ub^{K48R}. Because arginine at position 48 does not allow the conjugation of an additional Ub molecule, the incorporation of a Ub^{K48R} moiety should result in premature chain termination. A *H. polymorpha* strain (designated Ub.K48R) was constructed carrying the *Ub.K48R* gene under control of the strong, inducible alcohol oxidase (AO) promoter (P_{AOX}). At P_{AOX} inducing conditions, in this strain Ub^{K48R} will be produced in addition to the endogenously produced WT Ub.

Immunocytochemistry revealed that in glycerol-grown (moderate induction of the P_{AOX}; Fig. 1A) as well as methanol-grown (strong induction of the P_{AOX}; not shown) Ub.K48R cells, a major portion of the PTS1-containing peroxisomal matrix proteins AO (Fig. 1A) and catalase (CAT; data not shown) were mislocalized to the cytosol. In contrast, in identically grown WT control cells AO labeling was fully confined to peroxisomes (Fig. 1B). Because *H. polymorpha* requires fully functional peroxisomes to grow on methanol [23], the Ub.K48R strain displayed a strong defect in methanol growth as a result of the mislocalization of peroxisomal proteins. By contrast, the growth of

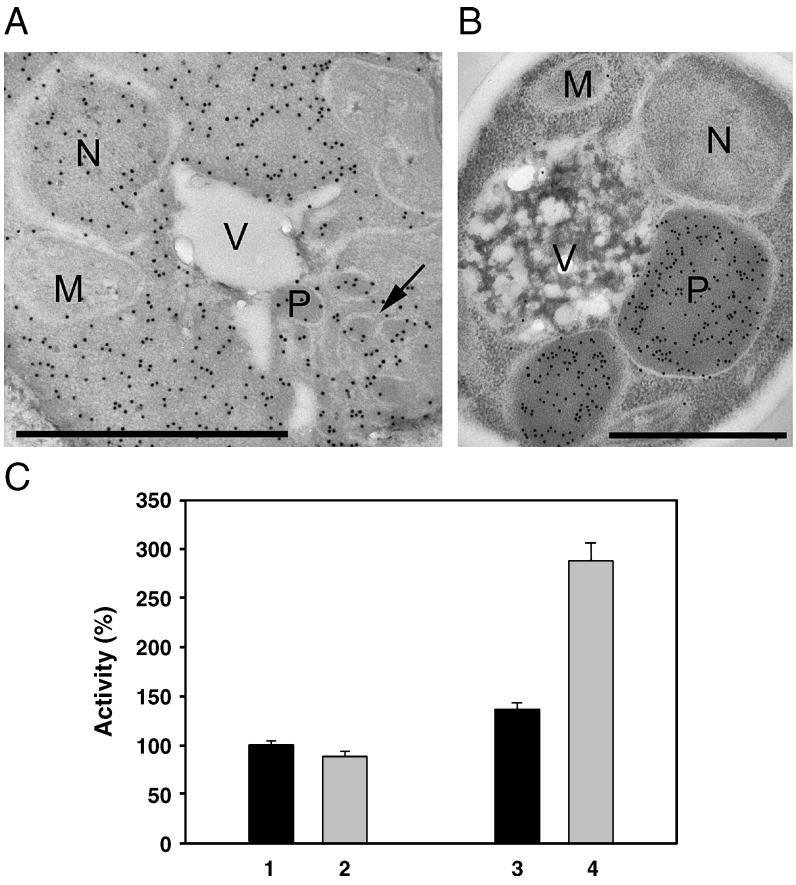

Fig. 1. Ub^{K48R} synthesis affects peroxisomal matrix protein import in *H. polymorpha*. (A, B) Immunocytochemistry of glycerol-grown Ub.K48R (A) and WT control cells (B) using anti-AO antibodies. Ub.K48R cells contain clusters of very small peroxisomes (arrow) that show a low rate of specific labeling relative to the bulk labeling on the cytosol. In WT control cells anti-AO specific labeling was confined to peroxisomal profiles (glutaraldehyde/anti-AO primary antibody/goat-anti-rabbit (GAR) conjugated to gold, uranylacetate). Key: M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. The bar represents 1 μm. (C) Ub^{K48R} synthesis stabilizes Ub-Leu-β-gal. Cells of *H. polymorpha* WT (black bars) and Ub.K48R cells (grey bars) synthesizing Ub-Leu-β-gal were grown at repressing conditions (glucose/methylamine; bars 1 and 2) to the mid-exponential growth phase and then shifted to P_{AOX} -inducing conditions to induce the synthesis of Ub^{K48R} (glycerol/methanol/methylamine; bars 3 and 4) for 12 h. The levels of β-galactosidase activities are expressed as percentages of the value determined for glucose/methylamine-grown WT cells (bar 1), which was arbitrarily set to 100%.

Ub.K48R cells on glycerol, the metabolism of which does not require functional peroxisomes, was similar to that of WT control cells (data not shown). This indicates that the levels of Ub^{K48R} produced in Ub.K48R cells were not lethal and had no major effect on essential cellular processes that involve polyubiquitination.

In *Saccharomyces cerevisiae* synthesis of Ub^{K48R} has previously been described to reduce polyubiquitination and stabilize certain proteins in vivo that are degraded by the proteasome [24]. Because this has not been reported yet for *H. polymorpha*, we analyzed the effect of Ub^{K48R} production in our model system. To this purpose we used Ub-Leu-β-gal as a model substrate, which in *S. cerevisiae* is very unstable according to the N-end rule [19]. The data presented in Fig. 1C show that the activity of β-galactosidase had increased significantly in glycerol/methanol-grown cells producing Ub^{K48R} relative to those observed in WT control cells. This indicates that P_{AOX}-driven Ub^{K48R} synthesis indeed reduces polyubiquitination in *H. polymorpha* under these growth conditions.

3.2. Ub^{K48R} synthesis does not affect PTS1 protein import in cells lacking Pex4p

If Ub^{K48R} production would interfere with Pex4p function, the synthesis of this mutant Ub is predicted to have no effect on PTS1 protein import in cells lacking Pex4p. This approach is feasible since in *H. polymorpha pex4* cells peroxisome biogenesis can be partially restored by the overproduction of Pex5p (*pex4::*P_{AOX}*PEX5*^{4c} cells; [10]). As shown in Fig. 2, peroxisomes containing significant amounts of AO are present in *pex4::* P_{AOX}*PEX5*^{4c} cells, both when Myc-tagged Ub (used as a control) or Myc-tagged Ub^{K48R} is being produced in these cells. These data suggest that Ub^{K48R} synthesis inhibits matrix protein import by obstructing the function of Pex4p.

3.3. Ub^{K48R} production results in reduced levels of Pex5p

To determine the steady state levels of peroxins and peroxisomal matrix proteins, cell extracts prepared from

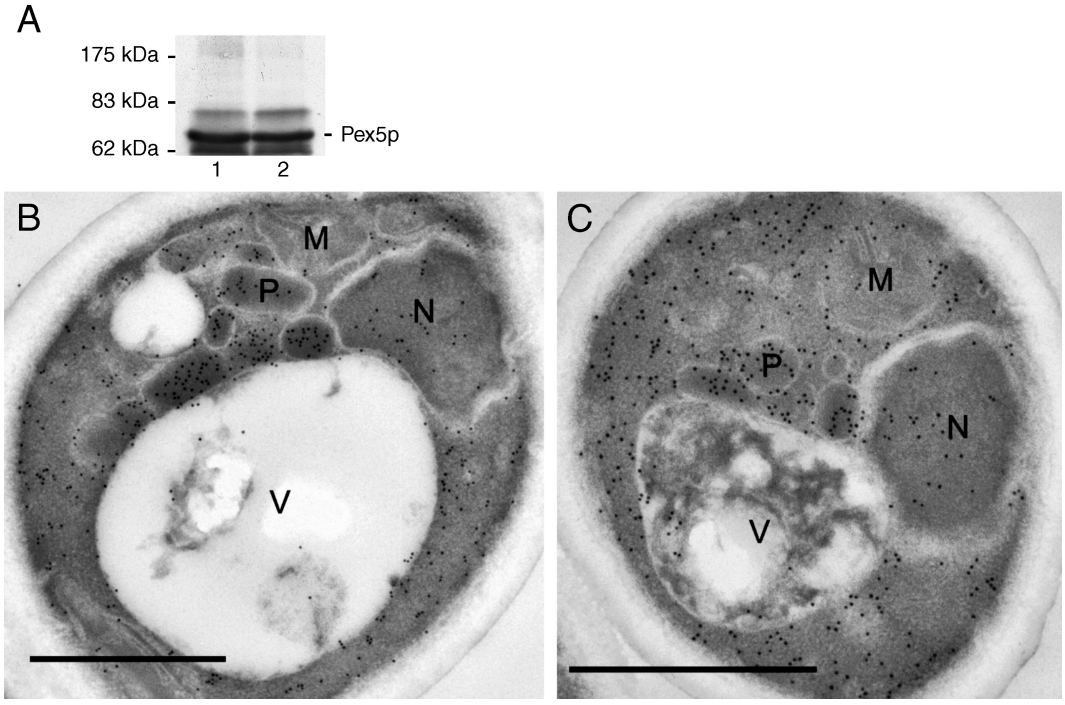

Fig. 2. Expression of *Ub.K48R* does not affect PTS1 matrix protein import in *pex4* cells. (A) Cells of *pex4*::P_{AOX}*PEX5* ::MycUb (lane 1) and *pex4*::P_{AOX}*PEX5* ::MycUb.K48R (lane 2) were grown on a methanol/glycerol containing medium for 16 h to maximally induce the expression of both the P_{AOX}-driven *PEX5* gene and the equally strong (data not shown) P_{DAS}-driven *MycUb* or *MycUb.K48R* genes. Western blot analysis using TCA extracts of both strains with anti-Pex5p antibodies revealed that similar Pex5p levels were present in both strains. (B, C) Immunocytochemical experiments show that in cells of both strains (B: *pex4*::P_{AOX}*PEX5* ::MycUb; C: *pex4*::P_{AOX}*PEX5* ::MycUb.K48R) a cluster of small peroxisomes of comparable size is visible that show identical anti-AO dependent labeling patterns in conjunction with a significant labeling on the cytosol. The identical peroxisomal labeling patterns indicate that the suppression of the PTS1 protein import defect in *pex4* cells as a result of *PEX5* overexpression is not disturbed by the simultaneous co-synthesis of $MycUb^{K48R}$ (aldehyde/anti-AO primary antibody/GAR-gold, uranylacetate). M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. Bar is 1 μm.

glycerol and methanol-grown Ub.K48R cells were analyzed by Western blotting using WT cells as controls. These studies revealed no significant changes in the levels of various matrix enzymes and peroxins in Ub.K48R cells as compared to WT cells (Fig. 3A) except for the PTS1 receptor Pex5p, which was strongly reduced in Ub.K48R cells. Closer analysis indicated that after a shift of Ub.K48R cells from glucose to glycerol-containing media, conditions that result in the induction of Ub.K48R synthesis, the amount of Pex5p rapidly decreased. By contrast, in WT control cells Pex5p levels increased after such a shift (Fig. 3C).

Also in *pex4* cells, the steady state Pex5p levels were significantly lower as compared to WT cells (Fig. 3B). Previously, we reported that in *H. polymorpha pex4* cells, Pex5p levels are somewhat higher than those observed in WT cells [10]. In that study, crude extracts were prepared using a glass bead cell breakage method. However, during recent years it has been recognized that Pex5p is extremely unstable in such lysates. It is thought that especially the cytosolic pool of Pex5p molecules is susceptible to proteolysis (cf. [25]). Thus, the Pex5p steady state levels determined with such lysates rather reflects the low amount of membrane-bound receptor molecules, which in *pex4* cells is higher than in WT cells [10]. To prevent any proteolytic degradation of Pex5p we now routinely precipitate whole cells with trichloroacetic acid (TCA) prior to cell breakage [20], resulting in a more accurate determination of total Pex5p levels.

For a more detailed analysis of the steady state Pex5p levels in WT and *pex4* cells, we also determined the levels of the PTS1 receptor upon a shift of cells from glucose to glycerol/methanol media (Fig. 3D). Under these conditions Pex5p was strongly induced in WT cells, whereas in *pex4* cells the level of Pex5p initially increased but subsequently decreased again.

A decrease in Pex5p levels was also observed when Ub^{K48R} was produced in *H. polymorpha pex7* cells, lacking the PTS2 receptor Pex7p, but not in *pex3* or *pex8* derivatives producing Ub^{K48R} (Fig. 4). This suggests that the decline in Pex5p levels in Ub.K48R cells requires the presence of the PTS1 protein import pathway, which is still functional in *H. polymorpha pex7* cells, but prohibited in *pex3* and *pex8* cells. The normal Pex5p levels observed in the *pex3*-Ub.K48R and *pex8*-Ub.K48R mutants as compared to the *pex3* and *pex8* controls, also indicate that Ub^{K48R} production does not influence the amount of Pex5p by affecting *PEX5* expression rates.

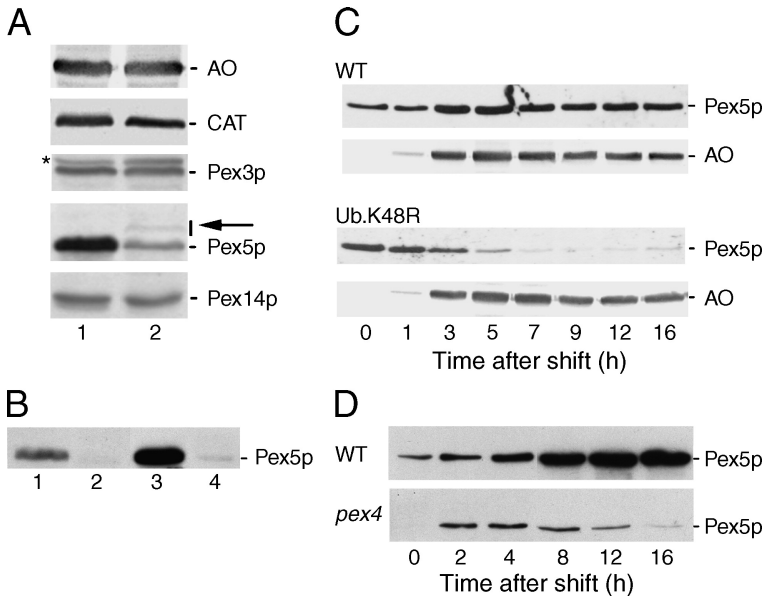

Fig. 3. Pex5p levels in *H. polymorpha* Ub.K48R and *pex4* cells. (A) *H. polymorpha* NCYC495 (lane 1) and Ub.K48R cells (lane 2) were induced for 18 h on methanol containing medium. Subsequently, TCA extracts were prepared and analyzed by Western blotting with equal amounts of protein loaded per lane. Blots were probed with specific antibodies against the indicated peroxisomal proteins. The peroxisomal enzyme AO and CAT, as well as the peroxins Pex3p and Pex14p, are found at similar levels in both strains. However, in Ub.K48R cells Pex5p levels are significantly reduced as compared to WT cells. Furthermore, an α-Pex5p immunoreactive protein band of higher Mr is sometimes observed in extracts of Ub.K48R (arrow), but not in WT cells. The asterisk indicates a non-specific protein band that is recognized by the α-Pex3p antiserum (see [33]). (B) Pex5p steady state levels in *pex4* cells. *H. polymorpha* NCYC495 (lanes 1 and 3) and *pex4* (lanes 2 and 4) cells were grown to mid-exponential growth phase on glucose (lanes 1 and 2) and glycerol/methanol (lanes 3 and 4). Samples were prepared for Western blotting with equal amounts of protein loaded per lane. Pex5p levels are significantly reduced in TCA extracts of *pex4* mutant cells as compared to WT cells. (C) Glucose-grown *H. polymorpha* WT and Ub.K48R cells were shifted from glucose to glycerol media to derepress P_{AOX}. Western blot analysis revealed that upon the shift, the synthesis of AO protein is readily induced in both strains. In WT cells Pex5p levels are induced at the first hours after the shift and then remain at a high level. In Ub.K48R cells, the induction of the synthesis of Ub^{K48R} results in a rapid decrease in Pex5p levels. (D) Glucose-grown *H. polymorpha* WT and *pex4* cells were shifted to glycerol/methanol-containing media. Western blots were prepared using anti-Pex5p antibodies. After the shift, Pex5p levels increase in WT cells. In *pex4* cells, Pex5p levels initially increase, but upon prolonged growth rapidly decrease again. Western blots were prepared from TCA extracts of the indicated strains and decorated with anti-Pex5p or anti-AO antibodies. Samples were taken at the indicated time points; equal amounts of protein were loaded per lane.

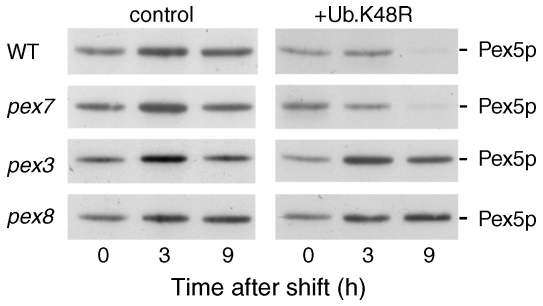

Fig. 4. The reduction in Pex5p steady state levels by the synthesis of Ub^{K48R} requires a functional PTS1 protein import pathway. The P_{AOX}-*Ub.K48R* expression cassette was integrated in 2 copies in the genomes of WT *H. polymorpha* and the mutant strains *pex3*, *pex7* and *pex8*. Glucose-grown cells of the transformants and the parental strains were shifted to glycerol to induce the synthesis of Ub^{K48R}. Samples were taken 3 and 9 h after the shift and prepared for Western blotting using anti-Pex5p antibodies. Equal amounts of protein were loaded per lane. In WT and *pex7* cells, in which the PTS1 pathway is operative, the expression of the *Ub.K48R* gene (strains Ub.K48R and *pex7*-Ub.K48R) results in strongly reduced Pex5p levels. In *pex3* and *pex8* cells Ub^{K48R} synthesis (strains *pex3*-Ub.K48R and *pex8*-Ub.K48R) has no significant effect on Pex5p levels.

3.4. Degradation of Pex5p occurs via the proteasome

To investigate whether the reduction in Pex5p levels in Ub.K48R and *pex4* cells was due to proteolysis, the effect of the proteasome inhibitor MG132 on Pex5p levels was determined. MG132 was added to Ub.K48R cells or *pex4* cells grown to the mid-exponential growth phase on glycerol. Western blots showed that upon incubation in the presence of MG132 significantly enhanced levels of Pex5p were detectable in cells of both strains, relative to untreated controls (Fig. 5A). A strong increase in Pex5p levels was not observed in WT cells upon the addition of MG132 (Fig. 5A).

Upon long exposure of Western blots prepared from crude extracts of Ub.K48R cells or *pex4* cells decorated with anti-Pex5p antibodies, an additional protein band of a higher apparent molecular weight was detected that had the expected molecular weight of a Pex5p–Ub conjugate (Fig. 5B). The molecular weight of this additional band slightly increased when a Myc-tagged version of Ub^{K48R} (in Myc-Ub.K48R cells) or Ub (in *pex4*-MycUb cells) was produced, indicating that these Pex5p bands indeed represented ubiquitinated Pex5p (Fig. 5B). Combined, these results

suggest that in *H. polymorpha* Ub.K48R and *pex4* cells a significant portion of Pex5p molecules become ubiquitinated and subsequently degraded by the Ub–proteasome pathway.

3.5. Pex5p degradation depends on lysine 21

The ubiquitination of proteins generally occurs at the ε-amine group of lysine residues present in the target protein. To determine which lysine residue in *H. polymorpha* Pex5p is involved in its degradation by the Ub–proteasome pathway, we inspected the amino acid sequence of Pex5p's from different yeast species for the presence of conserved lysine residues. We confined the comparison to the amino terminal half of these proteins, because this region is sufficient to import AO into peroxisomes of *H. polymorpha* [26]. In this region of *H. polymorpha* Pex5p, 4 lysines are present. Three of these are non-conserved residues, present in WxxxY/F motifs that are thought to be required for the binding of Pex5p to Pex14p [27]. A conserved, fourth residue, is located in the highly conserved extreme amino-terminus of Pex5p (Lys21; Fig. 6A). To study whether this lysine residue was involved in Pex5p ubiquitination, a mutation was introduced that resulted in the replacement of Lys21 by an arginine ($Pex5p^{K21R}$). The introduction of the mutated *PEX5* gene in a *H. polymorpha PEX5* deletion strain (PEX5.K21R) did not affect the growth of cells on methanol, indicating that $Pex5p^{K21R}$ normally functions in PTS1 protein import. Furthermore, Western blot analysis revealed that in this strain the steady state Pex5p levels were similar to those observed in WT cells (Fig. 6B, compare lanes 1 and 3). Notably, when Ub^{K48R} was produced in PEX5.K21R cells, the levels of $Pex5p^{K21R}$ did not decrease (Fig. 6B, compare lanes 2 and 4), indicating that Lys21 plays a crucial role in the degradation of Pex5p via the Ub/proteasome pathway. Similarly, we also observed that when the endogenous *PEX5* gene in *pex4* cells was replaced by a gene producing $Pex5p^{K21R}$, the mutant receptor was not degraded unlike the endogenous WT Pex5p (Fig. 6C).

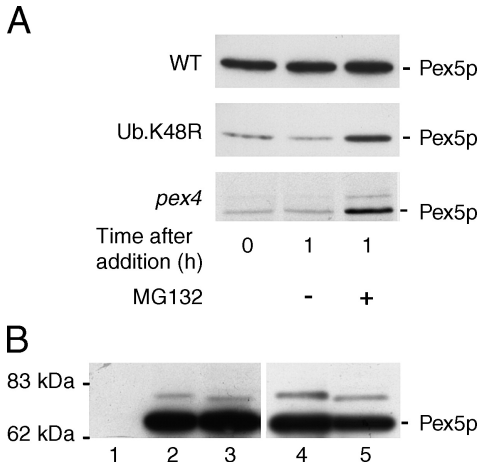

Fig. 5. Pex5p is degraded by the proteasome in Ub.K48R and *pex4* cells. (A) *H. polymorpha* WT, Ub.K48R and *pex4* cells were grown on glycerol to the exponential growth phase to induce the synthesis of Ub^{K48R}. Subsequently, the proteasome inhibitor MG132 was added. Samples, taken after 1 h of incubation in the presence of MG132, were prepared for Western blotting using anti-Pex5p antibodies. Cultures to which no MG132 was added were used as controls. Equal amounts of protein were loaded per lane. In Ub.K48R and *pex4* cells, but not in WT cells, the addition of MG132 results in strongly enhanced Pex5p levels indicating that Pex5p is massively degraded by the proteasome in Ub.K48R and *pex4* cells. (B) *H. polymorpha pex5* (lane 1), MycUb.K48R (lane 2), Ub.K48R (lane 3), *pex4*-MycUb (lane 4) and *pex4* (lane 5) cells were grown on glycerol/methanol for 4 h. The synthesis of Ub^{K48R} (size: 8.5 kDa) in strain Ub.K48R results in the formation of a small amount of an anti-Pex5p immunoreactive protein band of higher Mr (approx. 8.5 kDa higher than the main Pex5p band; lane 3), which is shifted to an even higher Mr when $MycUb^{K48R}$ (approx. 10 kDa higher) is produced (lane 2). Similarly, an additional protein band of higher Mr (approx.+8.5 kDa) cross-reacting with anti-Pex5p antibodies is present in *pex4* cells (lane 5), which is shifted to a higher Mr (+10 kDa) when MycUb is produced (lane 4). Western blot of TCA extracts decorated with anti-Pex5p antibodies. Equal amounts of protein were loaded per lane.

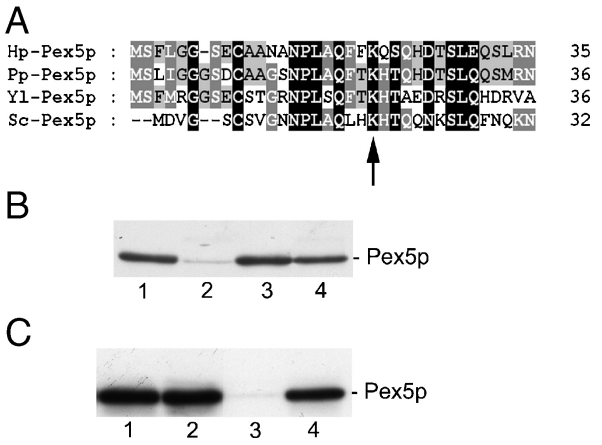

Fig. 6. The N-terminal region of yeast Pex5p's contains a conserved lysine residue that is involved in ubiquitination. (A) The conserved N-terminal regions of Pex5p's from *H. polymorpha* (Hp, SwissProt Q01495), *Pichia pastoris* (Pp, Swissprot P33292), *Yarrowia lipolytica* (Yl, Swissprot Q99144) and *S. cerevisiae* (Sc, SwissProt P35056) were aligned using the Clustal_X programme. Gaps were introduced to maximize the similarity. Residues that are similar in all proteins are shaded black. Similar residues in three of the proteins are shaded dark grey while those that are similar in two of the proteins are shaded light grey. The arrow indicates the *H. polymorpha* Lys21 residue that is conserved in all four sequences. (B) *H. polymorpha* WT (lane 1), MycUb.K48R (lane 2), PEX5.K21R (lane 3) and PEX5.K21R MycUb.K48R (lane 4) cells were grown on glycerol/methanol media. The mutation of the Lys21 residue in Pex5p has no effect on the steady state levels of the PTS1 receptor in *H. polymorpha* WT cells, but stabilizes the receptor in cells producing $MycUb^{K48R}$. (C) *H. polymorpha* WT (lane 1), PEX5.K21R (lane 2), *pex4* (lane 3) and *pex4* PEX5.K21R (lane 4) cells were grown on glycerol/methanol media. The mutation of the Lys21 residue in Pex5p stabilizes the protein in *pex4* cells. (B,C) Western blots of TCA extracts decorated with anti-Pex5p antibodies. Equal amounts of protein were loaded per lane.

4. Discussion

Pex4p, one of the first identified peroxins [8], is involved in peroxisomal matrix protein import. This peroxin has been

shown to be a true Ub-conjugating enzyme (E2), because its active site cysteine is essential for its function in peroxisome biogenesis and can bind Ub via a thioester bond [8,9]. Pex4p is presumably recruited to the outer surface of the peroxisomal membrane by the integral membrane protein Pex22p, which is essential for the function and stability of Pex4p [11].

So far, the principles of Ub-conjugation by Pex4p in the process of peroxisomal protein translocation are still an enigma. Both the predicted E3 Ub ligase, which is important for the recognition of the substrate, and the substrate itself have not been identified yet. It has been suggested that Pex5p may be the actual substrate of Pex4p. We show here that *H. polymorpha* Pex5p can indeed be ubiquitinated. Remarkably, however, this process does not require the function of Pex4p.

It was also still unknown whether Pex4p mono- or polyubiquitinates its yet-unknown substrate. In this paper we present the first indication that the function of *H. polymorpha* Pex4p involves polyubiquitination, because interference with Lys48-linked polyubiquitination results in a dramatic effect on PTS1 protein import, similar as that observed in *H. polymorpha pex4* cells. Moreover, this effect was not evident in cells lacking Pex4p, in which the peroxisome biogenesis defect was largely suppressed.

Previous observations indicated that Pex4p functions at a late stage in PTS1-matrix protein import, namely recycling of the PTS1-receptor Pex5p to the cytosol. We demonstrated in *H. polymorpha pex4* cells that the overproduction of Pex5p partly suppressed the PTS1 protein import defect. It was noted that in such strains significant amounts of Pex5p accumulated in the organellar matrix [10], suggesting that the release of Pex5p from the organelle to allow another round of Pex5p-mediated import is disturbed in *H. polymorpha pex4* cells. Similarly, the results of an epistasis analysis on *P. pastoris pex* mutants indicated that Pex4p and Pex22p play a role in Pex5p recycling [7]. Recent data in *S. cerevisiae* are in line with this view [28,29].

The instability of Pex5p has been reported before for *P. pastoris pex4*, *pex22*, *pex1p* and *pex6* mutants [7,11], human cell lines defective in Pex1p or Pex6p [30] and *Arabidopsis thaliana pex6* cells [31]. Our current data also revealed highly reduced steady state Pex5p levels in *H. polymorpha pex4* cells and cells overproducing Ub^{K48R}. Moreover, we show that this dramatic decrease in Pex5p levels is due to degradation of the protein via the proteasome, a process that appears to involve Lys21 in HpPex5p.

In all model systems under study, Pex5p instability is invariably observed in (mutant) cells in which the initial stages of PTS1 protein import (docking, translocation) are still functional, but later stages are disturbed. We indeed found that in *H. polymorpha* degradation of Pex5p as a result of Ub^{K48R} synthesis did not take place in *pex3* and *pex8* mutants, in which Pex5p cannot reach the late stage of the import cycle. Based on these findings we propose that *H. polymorpha* Pex5p molecules that are not efficiently recycled to the cytosol are removed by the Ub–proteasome pathway (for a hypothetical model see Fig. 7).

Recently, it was reported that in *S. cerevisiae* a defect in Pex5p recycling (in *pex1*, *pex6*, *pex15*, *pex4* and *pex22* null mutants) does not result in a significant reduction of Pex5p levels [28,29]. However, in the cells of these mutant strains a portion of the Pex5p molecules became ubiquitinated. Moreover, this process was shown to be independent of Pex4p, but required the function of yet another ubiquitin-conjugating enzyme, Ubc4p [28,29].

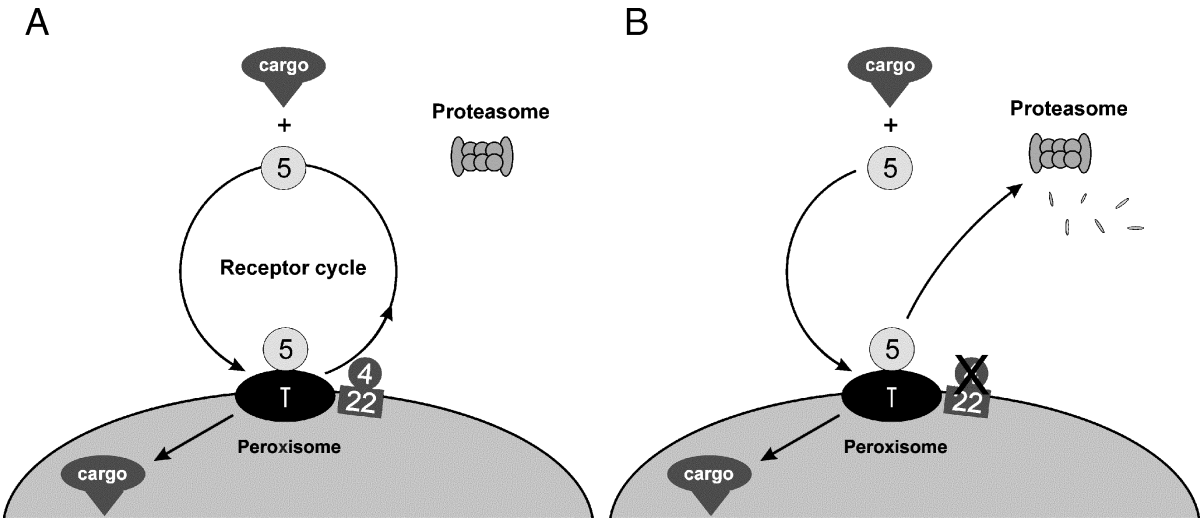

Fig. 7. Hypothetical model of the Pex5p mediated protein import cycle and Pex5p degradation. (A) Pex5p-mediated PTS1 protein import. Newly synthesized PTS1 proteins are recognized by Pex5p in the cytosol. After the import and release of the cargo, Pex5p recycles to the cytosol. This process involves polyubiquitination and the function of Pex4p, which is recruited to the peroxisomal membrane by Pex22p. T-putative translocase. (B) Inactivation of Pex4p results in Pex5p degradation. Upon the obstruction of Pex4p function (e.g. in a *pex4* mutant, upon synthesis of Ub^{K48R} or at non-optimal physiological conditions) the recycling of Pex5p is hampered. These non-recycled Pex5p molecules are ubiquitinated and degraded by the proteasome.

In exponential methanol-grown *H. polymorpha* WT cells, the level of Ub-conjugated Pex5p is invariably below the limit of detection (Kiel and Van der Klei, unpublished results). Moreover, the mutation of the conserved Lys21 residue in the N-terminus of Pex5p, which is important for the degradation of the protein via the Ub–proteasome pathway, does not affect the growth of cells on methanol. Our interpretation of these data is that ubiquitination of Pex5p at Lys21 is important to remove those Pex5p molecules from the translocation site that are not efficiently recycled to the cytosol (Fig. 7). In WT *H. polymorpha* cells grown at physiologically optimal growth conditions, such a situation may occur relatively seldom. However, at poor physiological conditions (e.g. ATP depletion, very low growth rates), such a mechanism may be essential to retain a functional peroxisomal translocation machinery in WT cells. In specific *H. polymorpha* mutants that show reduced Pex5p recycling this process is apparently very active in degrading Pex5p molecules, explaining why Pex5p is very unstable in such cells (Fig. 7).

In line with our data in *H. polymorpha*, also in *S. cerevisiae* ubiquitinated Pex5p was only observed in a specific mutant, but not in WT cells [28,29]. Moreover, the deletion of *UBC4* in *S. cerevisiae*, which is responsible for the Pex5p ubiquitination in mutant strains defective in receptor recycling, did not affect peroxisome biogenesis, as indicated by the normal peroxisomal localization of GFP-SKL and normal growth of cells on oleic acid [28,29]. Notably, Platta and co-workers observed a minor defect in peroxisomal protein import in cells of a *UBC4 UBC5* double deletion strain, which was explained by the partially overlapping functions of Ubc4p and Ubc5p in *S. cerevisiae* [28]. To our view this effect suggests that Ubc4p/Ubc5p dependent Pex5p ubiquitination may be of little importance for peroxisomal protein import under optimal physiological conditions in *S. cerevisiae* WT cells. Although we cannot exclude the alternative explanation, that Ubc4p/Ubc5p-dependent Pex5p ubiquitination represents a genuine stage in the Pex5p receptor cycle in WT *S. cerevisiae* cells [28], such a pathway is very unlikely to occur in *H. polymorpha*, since the mutation of the Lys21 residue in Pex5p that is essential for Pex5p ubiquitination does not affect PTS1 protein import in this yeast.

Our data suggest that poly-Ub chains via Lys48 of Ub is related to the function of *H. polymorpha* Pex4p. One explanation may be that Ub conjugation by Pex4p involves Lys48-linked poly-Ub chains that are conjugated to an as yet unknown protein substrate. The production of Ub^{K48R} in *H. polymorpha* WT cells resulted in the presence of a mixture of normal and mutant Ub molecules in the cells. Indeed, the level of Ub^{K48R} that was obtained in the constructed *H. polymorpha* strain during the growth of cells on glycerol/methanol mixtures resulted in the reduction of the degradation of a model substrate by the Ub–proteasome pathway (see Fig. 1C). However, a full block in the proteolytic function of the Ub–proteasome pathway, which should be lethal to cells, was clearly not achieved, because the cells still grew on glycerol at the same growth rate as WT cells. The obtained Ub^{K48R} levels however had a significant effect on peroxisomal matrix protein import at the same growth conditions.

The identification of the substrate of Pex4p and the predicted E3 that acts in conjunction with Pex4p are of major importance to allow to design experiments that may provide further insights in the function of the proposed poly-ubiquitination process. Recently, it has been suggested that the RING finger protein Pex10p may represent this E3, based on physical interaction between *S. cerevisiae* Pex4p and Pex10p [32] and the fact that RING finger motifs are important components of certain E3 ligases. Experimental proof for this assumption will be of great importance for further studies to elucidate the function and substrate of Pex4p.

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